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TITLE:

Myocardial ischemia/reperfusion upregulates the transcription of the Neuregulin1 receptor ErbB3, but only postconditioning preserves protein translation: role in oxidative stress.

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ABSTRACT:

Neuregulin1 (Nrg1) and its receptors ErbB are crucial for heart development and for adult heart structural maintenance and function and Nrg1 has been proposed for heart failure treatment. Infarct size is the major determinant of heart failure and the mechanism of action and the role of each ErbB receptor remain obscure, especially in the post-ischemic myocardium. We hypothesized that Nrg1 and ErbB are affected at transcriptional level early after ischemia/reperfusion (I/R) injury, and that the protective postconditioning procedure (PostC, brief cycles of ischemia/reperfusion carried out after a sustained ischemia) can influence this pathway. The Langendorff's heart was used as an *ex-vivo* model to mimic an I/R injury in the whole rat heart; after 30 min of ischemia and two hours of reperfusion, with or without PostC, Nrg1 and ErbB expression were analyzed by quantitative real-time PCR and Western blot. While no changes occur for ErbB2, ErbB4 and Nrg1, an increase of ErbB3 expression occurs after I/R injury, with and without PostC. However, I/R reduces ErbB3 protein, whereas PostC preserves it. An *in vitro* analysis with H9c2 cells exposed to redox-stress indicated that the transient over-expression of ErbB3 alone is able to increase cell survival (MTT assay), limiting mitochondrial dysfunction (JC-1 probe) and apoptotic signals (Bax/Bcl-2 ratio). This study suggests ErbB3 as a protective factor against death pathways activated by redox stress and supports an involvement of this receptor in the pro-survival responses.

Keywords: ErbB3; Neuregulin1; Postconditioning; ErbB; Ischemia/reperfusion; Nrdp1

1. Introduction

Neuregulin1 (Nrg1) is a signalling protein belonging to the Epidermal Growth Factor (EGF) gene family that mediates various cellular processes, such as cell growth, survival, migration, in different cell types, through the ErbB receptors. Nrg1/ErbB system is essential for a correct cardiac development, as demonstrated by the lethal effects of its depletion at embryonic stages [1,2]. Furthermore, it is now clear that also in adult heart this signalling plays a critical role in the normal function as well as in ischemia or other pathological conditions [3,4]. In the NRG1 gene, alternative splicing gives rise to different isoforms and, in adult heart, cardiac microvascular endothelial cells (EC) express soluble Nrg1 isoforms (type I and type II), both alpha and beta variants [5], which stimulate cell survival and growth [6], glucose up-take [5], protein synthesis and “hypertrophic” gene expression [7]. Nrg1 mediated the cross-talk between EC and cardiomyocytes that express ErbB receptors [8], and the deletion of Nrg1 from EC increases the infarct area and the number of TUNEL positive cells after ischemia and reperfusion (I/R) injury [9].

ErbB receptors belong to the tyrosine kinase receptor family, and work as dimers. Among the four ErbB receptors only ErbB3 and ErbB4 can bind directly Nrg1; ErbB3 signals only as heterodimer and ErbB2 is the preferred partner for heterodimerization, while ErbB4 can form both homo and heterodimers. Initially only ErbB1, ErbB2 and ErbB4 were thought to be expressed in adult heart. In 2011, Camprecios and colleagues demonstrated that mouse post-natal cardiomyocytes express a functional ErbB3 protein that is localized mainly in the outer areas of T-Tubules with a non-uniform distribution [10]. Although a methylation of ERBB3 gene has been observed in human dilated cardiomyopathy [11], the function of ErbB3 in adult heart remains still unknown. Recently, it has been demonstrated that a E3 ligase known as “neuregulin receptor degradation protein-1” (Nrpd1), which targets specifically ErbB3 [12], is upregulated after I/R injury. The mouse model over-expressing Nrpd1 is characterized by higher infarct size, increased TUNEL-positive nuclei and inflammatory cells [13]. It was postulated that Nrpd1 is a pro-apoptotic signal in heart during I/R injury and that its action is mediated principally by the degradation of ErbB3.

For its pro-survival effect Nrg1 has been proposed as a potential drug for heart failure treatment. Several pre-clinical studies in rat or mouse models of heart failure and two clinical trial [14,15] demonstrated that intravenous administration of recombinant soluble Nrg1 improved cardiac contractility and relaxation [16,17], left ventricular remodelling [18],

decreased apoptosis [19] and attenuated mitochondrial dysfunction [18]. However, the molecular bases of this beneficial effect remain unclear.

It has not yet been investigated whether Nrg1/ErbB system is modulated by existing therapeutic strategies aimed to protect the heart against ischemia and reperfusion injury. One of the most interesting cardioprotective strategy is the so-called “Postconditioning” (PostC, *i.e.*, brief cycles of ischemia/reperfusion carried out after a sustained ischemia) [20–23], which can reduce the infarct size in animal models [24,25] and humans [26–28]. The cardioprotective mechanisms activated by PostC comprise the activation of multiple pathways, including the so-called RISK (Akt/ERK/GSK3- β) and SAFE (JNK/STAT3) pathways [29–31]. Since these pathways are downstream of the Nrg1/ErbB system [29, 30, 31], which in PostC context is scarcely investigated, we studied how this system is influenced by I/R and the PostC procedures.

We analysed the early changes in Nrg1/ErbB system following ischemia/reperfusion injury in isolated rat heart, with or without the PostC treatment. Moreover, we investigated in a cardiomyocyte cell line whether the ErbB3 receptor plays a role in oxidative stress.

Overall results suggest that this receptor can be involved in the myocardial response to ischemia and reperfusion challenge. In particular, we confirmed that ErbB3 is expressed in rat adult heart and we demonstrated, for the first time, that its expression is upregulated in post-ischemic heart, suggesting that ErbB3 protein might play a role in PostC and oxidative injury limitation.

2. Methods

2.1 Animals

Male Wistar rats (n=18, 5–6 month old, body weight 450–550 g) were purchased from Harlan (Bresso, MI, Italy). Animals received care in compliance with the Italian law (DL-116, January 27, 1992) and with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. These laws are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2 Langendorff's heart model

Methods for Langendorff's isolated rat hearts were similar to those previously described [32,33]. In brief, each animal was weighed and treated with heparin (800 U/100 g b.w., i.m.). Then, 10 min afterwards, animal was sacrificed, the heart was rapidly excised and placed in ice-cold buffer solution and weighed. Isolated hearts were retrogradely perfused at constant flow (9 ± 1 ml/min/g) with oxygenated Krebs–Henseleit buffer (127 mM NaCl, 17.7 mM NaHCO₃, 5.1 mM KCl, 1.5 mM CaCl₂, 1.26 mM MgCl₂, 11 mM D-glucose) (Sigma-Aldrich, St. Louis, MO, USA) gassed with 95% O₂ and 5% CO₂, paced at 280 bpm and kept in a temperature-controlled chamber (37°C).

2.3 Experimental Protocols

After 20 min of stabilization, hearts were randomly divided in three groups: (1) Control group (Sham), hearts were subjected to 150 min perfusion only; (2) I/R group, hearts underwent 30 min of global ischemia and then a period of 120 min full reperfusion; (3) PostC group, after 30 min ischemia, hearts underwent a PostC protocol (5 cycles of 10 sec reperfusion and 10 sec of global ischemia) then a period of 120 min full reperfusion [24].

2.4 Myocardial Injury

2.4.1. Infarct size

Immediately after reperfusion each heart was quickly removed from the perfusion apparatus and the apical part of the heart was collected and frozen for biomolecular analysis. The remaining basal part of the left ventricle was dissected into 2–3 mm circumferential slices.

Following 20 min of incubation at 37°C in 0.1% solution of nitro-blue tetrazolium in phosphate buffer, unstained tissue was carefully separated from stained tissue by an independent observer. The unstained tissue represents the amount of death cells, the stained tissue represents the viable cells. A gravimetric method was used: the unstained mass was weighed and then expressed as a percentage of left ventricular mass [24,32,33]. Reagents necessary to assess myocardial infarction were purchased from Sigma (USA).

2.4.2. Lactate dehydrogenase analysis

Since in isolated rat hearts PostC is known to reduce the production of lactate dehydrogenase (LDH) during reperfusion, the release of this enzyme was tested. Samples of coronary effluent (2 ml) were collected with a catheter inserted into the right ventricle via the pulmonary artery. Samples were collected immediately before ischemia during reperfusion. Thereafter samples were collected every 20 min until the end of reperfusion. LDH release was measured as previously described and data are expressed as cumulative values for the entire reperfusion period [24,32].

2.5. RNA isolation and cDNA preparation

Total RNA was extracted from half apex from each heart, previously dissociated by pestles, with TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Glycogen (5 µg) was added as a carrier to facilitate RNA precipitation. For each sample 1µg total RNA was reverse transcribed (RT): samples were warmed at 65 °C for 5 minutes and the reaction was carried out in 20 µl containing: 1 × RT-Buffer (Life Technologies), 0.1 µg/µl bovine serum albumin (BSA, Promega, Fitchburg, WI, USA), 0.5 mM dNTPs, 7.5µM random decamers (Life Technologies), 5mM DTT (Life Technologies), 40 U RNase Out Inhibitor and 200U SUPERScript III Rev transcript (Life Technologies,18080044). The reaction was performed for 10 min at 25 °C, 90 min at 50 °C, 15 min at 70 °C. The obtained cDNA was diluted 10 folds with water and stored at -20 °C.

2.6. Quantitative real-time PCR (qRT-PCR) analysis

Quantitative real-time PCR analysis was performed in a 7300 real-time PCR system (Applied Biosystems). The reaction was carried out in 20 µl containing 5 µl diluted cDNA (corresponding to 25 ng of starting RNA), 1x Sybr Green PCR Master Mix (Bio-Rad, Hercules,

CA, USA) and 300nM forward and reverse primers. For each sample a technical triplicate was performed. All primers were designed to amplify specific isoforms of ErbB receptors or Neuregulin-1 and Nrdp1 (Table 1). The reaction was carried out with the following protocol: 30 seconds (sec) at 95°C, 40 cycles of denaturation at 95 °C for 15 sec followed by primer annealing and elongation at 60 °C for 1 minute. Dissociation curve was always analysed to control the quality of the reaction.

Data were analyzed by the $\Delta\Delta C_t$ relative quantification method normalizing to the geometric average of two housekeeping genes, Ubiquitin C (UbC) and Hypoxanthine guanine phosphoribosyltransferase (HPRT). We determined the difference between C_t values of target and housekeeping gene (ΔC_t), the difference between the ΔC_t values of the samples and the ΔC_t mean value of control sample was then calculated ($\Delta\Delta C_t$). The normalized relative quantity (NRQ) was determined using the formula: $NRQ = 2^{-(\Delta\Delta C_t)}$. NRQ value >1 reflects an increasing of target gene expression, NRQ<1 reflects a decreasing of target gene expression [34]. The analysis was carried out in biological quintuplicate (for each experimental group n=5) and results were expressed as mean \pm standard deviation.

2.7. Total protein extraction and Western Blot analysis

Total proteins were extracted from heart apexes with boiling Laemmli buffer (2.5% SDS, 0.125M Tris-HCl pH 6.8) dissociating the tissue with pestles and incubating the protein extract at 100°C for 3 minutes. The BCA method was used to quantify proteins and the same amount of protein for each sample was loaded for SDS-PAGE analysis. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, 162-0093). Primary antibodies used are: rabbit polyclonal anti-ErbB3 (working dilution, w.d., 1:1000, sc-285, Santa Cruz, Santa Cruz, CA, USA), anti-ErbB2 (w.d. 1: 1000, sc-284, Santa Cruz), anti-alpha sarcomeric actinin (w.d. 1:2500, A7811, SIGMA), anti-phospho S6 ribosomal protein (w.d. 1:2000, 4858, Cell Signaling). The secondary antibodies used are: ECL™ anti-rabbit IgG (w.d. 1:40000, NA934, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and ECL™ anti-mouse IgG (w.d. 1:40000, NA931, GE Healthcare). The western blot quantitative analysis was performed using the Image J program.

2.8. Cell culture and in vitro experiments

For the *in vitro* analysis we used H9C2 cells, a rat myoblast cell line provided by American Collection of Cell Cultures (ATCC® CRL-1446™ Milan Italy). Cells were cultured in Dulbecco's Modified Eagle's Medium Nutrient mixture F-12 HAM (DMEM, D8437, SIGMA) supplemented with 10% fetal bovine serum (FBS, GIBCO, Life Technologies) and 1% (v/v) streptomycin/penicillin (Wisent Inc, Quebec, Canada).

To express the ErbB3 protein, the expression vector pcDNA3-B3, kindly provided by Dr. John G. Koland (Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, IA) was used [35]. For transient transfections, Lipofectamine 2000 (Life Technologies) was used following manufacturer's instruction. Briefly, cells were grown in 20cm² dishes, then medium was changed to Optimem (Life Technologies) and cells were transfected with 4μg plasmid DNA and 4μl Lipofectamine 2000. Control mock transfections were carried out with the empty vector pcDNA3. After 16 hours the medium was changed to normal culture medium and after other 24 hours cells were detached and left to grow in 60 mm dish.

For the oxidative stress we treated H9C2, H9C2-ErbB3 and H9C2-mock cells with hydrogen peroxide solution (H₂O₂, H1009, SIGMA), used for 2 hours, in a range of concentration 100 μM-300 μM to obtain 40-50% of cell mortality. Untreated H9c2 were compared to H9c2 exposed to 50 ng/ml Nrg1-β1 (recombinant human NRG1-β1/HRG1-β1 extracellular domain, # 377-HB-050, R&D Systems, Minneapolis, MN).

A pre-treatment of the cells with 50 ng/ml Nrg1 for 2 h was also performed, followed by the oxidative stress with hydrogen peroxide solution. Cell survival rate was obtained using the thiazolyl blue tetrazolium bromide (MTT, M2128, SIGMA) assay, following producer's instruction. Cell survival rate of each cell group were analysed separately. For each independent experiment, data for each condition were calibrated to the control ("no stress") sample.

For Bax and Bcl-2 analysis, cells were seeded on 60 mm dish and exposed to oxidative stress as described above. After 3h of reperfusion (cells in medium with 2% of fetal bovin serum) protein were extracted with boiling Laemmli buffer. Primary antibodies used are: anti-Bax (w.d. 1:300, sc-23959, Santa Cruz, CA, USA); anti-Bcl-2 (w.d. 1:500, sc-492, Santa Cruz, CA, USA); anti-β actin (w.d. 1:4000, #A5316, Sigma, Germany).

JC-1 fluorescent probe (Molecular Probes, Eugene, OR, USA) was used to measure loss of mitochondrial transmembrane potential after the oxidative stress protocol. In brief, sub-

group of cells after 1 hour of reperfusion were incubated with medium containing JC-1 (10 µg/ml) at 37°C for 20 min and then washed twice with PBS. Fluorescent values were acquired using a GloMax-Multi Detection System (Promega Corporation, Madison, WI, USA) with 485 nm and 530 nm as the green excitation and emission wavelengths, respectively, and 535 nm and 590 nm as red excitation and emission wavelengths, respectively. The ratio between red and green JC-1 fluorescence was taken as an index of mitochondrial membrane potential [36].

2.9. Statistical analysis

Statistical analysis was performed using IBM SPSS program. For *real-time* PCR data and *in vitro* data Student's t-Test was used. For protein quantitative analysis One Way Anova plus Fisher's LSD (Least Significant Difference) or plus Bonferroni Post Hoc test was used. Data are presented as mean ± SD. A probability value lower than 0.05 was considered as statistically significant.

3. Results

Taking advantage of the *ex vivo* Langendorff's heart model, we investigated the changes that occur in Nrg1 and ErbB expression in heart tissue following a global ischemia/reperfusion challenge (I/R group), or following the postconditioning procedure (PostC group). Infarct mass analysis confirmed the effectiveness of PostC procedure in reducing ischemia/reperfusion injury (48±16% vs 29±3.5% of risk area, in I/R and PostC respectively; $p<0.05$). LDH analysis corroborated the infarct size data (LDH release was 381±110 in I/R group and 188±80 IU in PostC hearts; $p<0.05$). The molecular analysis was carried out on five animals for each condition.

The expression of Nrg1 and ErbB receptors in the left ventricle was analysed using qRT-PCR; the mRNA level in I/R hearts was compared with the mRNA level observed in PostC hearts. Data show that the four ErbB receptor genes are transcribed in adult rat heart (Fig. 1). After 30 min ischemia and 2 hours reperfusion, the mRNA levels of ErbB1 and ErbB2 did not differ significantly from the Sham (Fig. 1a), regardless of PostC procedure. Similar results were obtained for the four ErbB4 isoforms (Fig. 1b). Conversely, ErbB3 mRNA (Fig. 1a) displayed a 2.8 fold increase after I/R ($p<0.05$ compared to the Sham) and a 2.6 fold increase after PostC ($P=0.081$ with respect to Sham).

Nrg1 transcription was also investigated, discriminating between the two isoforms alpha and beta. qRT-PCR analysis did not reveal changes in transcription among samples, as shown in Fig. 1c.

The analysis was then shifted from the mRNA level to the protein level, to investigate ErbB3 protein expression. The western blot analysis confirmed the expression of ErbB3 protein in rat adult heart (Fig. 2a). A quantitative analysis revealed an appreciable downregulation ($p=0.063$) of ErbB3 in I/R samples compared to the PostC (Fig. 2a), in partial contrast with mRNA analysis. We then analysed a marker of protein translation, namely phospho-S6 protein (p-S6), a subunit of ribosome S40 (when S6 subunit is phosphorylated the translation is permitted). The analysis revealed that I/R samples have a lower level of p-S6 when compared to PostC ($p=0.012$, Fig. 2a). Yet in PostC sample p-S6 was upregulated respect to the Sham ($p<0.05$, Fig. 2a). Furthermore, we checked the expression of Nrdp1, the E3 ligase targeting ErbB3, and we found that in I/R group the Nrdp1 mRNA level is significantly higher compared to Sham (2.6 ± 0.9 fold increase, $p<0.05$) but not in PostC group (Fig. 2b). Nrdp1 protein levels were analyzed in a western blot assay: the protein is downregulated in PostC group ($p=0.041$), but not in I/R group, respect to the Sham group (Fig 2c). A Student's t-test between PostC and I/R group reveals a statistically relevant difference ($p< 0.05$). These data together could explain the lower amount of ErbB3 protein in I/R samples despite the upregulation of ErbB3 mRNA expression. Intriguingly, PostC preserves ErbB3 protein.

Data regarding Nrdp1 suggest that ErbB3 protein absence contributes to the I/R damage and that the presence and stability of ErbB3 protein could protect the tissue. So we examined *in vitro* the ability of ErbB3 receptor to defend cells from oxidative damage. H9c2 cells were used as a model; because they express ErbB2 and soluble Nrg1, and a barely detectable ErbB3 and ErbB4 (data not shown), they were transiently transfected for the expression of ErbB3 receptor, and were subjected to oxidative stress with hydrogen peroxide. In non-transfected cells the administration of Nrg1, before or simultaneously to the oxidative stress, had no effect on cell survival rate in a MTT test (Fig 3a). The expression of ErbB3 was confirmed with a western blot, together with ErbB2 protein constitutively expressed by H9c2 (Fig. 3b). Results show that ErbB3-expressing cells tolerate oxidative stress better than control cells (H9c2-mock) in each treatment evaluated. Nevertheless, the addition of Nrg1 to the medium did not increase cell survival rate in H9c2-ErbB3 and H9c2-mock cells (Fig. 3c). We analysed the

Bax/Bcl-2 ratio as an indicator of cell apoptosis. We found that in H9c2-ErbB3 cells the ratio, after oxidative stress, remains lower respect to H9c2-mock cells (Fig. 3d,e). Moreover, the analysis of JC-1 fluorescent probe confirms that ErbB3 expression in H9c2 cells significantly limits the reduction of mitochondrial membrane potential with respect to H9c2-mock cells in all treatment performed (Fig 3f).

4. Discussion

To determine whether ErbB receptors and Nrg1 are early affected by the ischemia-reperfusion injury and PostC treatment, we analyzed mRNA and protein expression levels in samples collected from the rat heart injured by global I/R, in a Langendorff's model which, like all experimental paradigms, has advantages and disadvantages [37]. For instance, Langendorff's model allows to perform a global ischemia, subjecting the heart to a homogeneous stress insult, and to remove unwanted blood cells, protein and mRNA from the myocardium, thus allowing to perform a "clean" molecular analysis.

The expression analysis of ErbB receptors reveals an upregulation of ErbB3 mRNA after ischemic injury, while the expression of the other ErbB receptors is not perturbed. As far as we know, this is the first time that ErbB3 mRNA is shown to be regulated in post-ischemic heart. Since no statistic differences in ErbB3 expression were detectable between PostC and I/R samples, it is likely that this upregulation is mainly a direct early response to the I/R, not modifiable with PostC. Yet, PostC can positively affect the translation machinery, thus preserving ErbB3 protein synthesis.

It is known that ErbB2 and ErbB4 mRNA expression is reduced in human and rat heart after heart failure [3,38,39]. This was observed also in diabetic animals, showing cardiac dysfunctions [40]. It was proposed that the downregulation of ErbB2 and ErbB4 is a late characteristic of the chronic heart injury. Here we focused on the early changes occurring in gene expression after I/R challenge and PostC, suggesting that in the early-phase ErbB2 and ErbB4 are not regulated at transcriptional level.

ErbB3 mRNA is upregulated after I/R, while for ErbB3 protein only a tendency to downregulation in I/R group, but not in PostC samples, can be observed. This tendency is considered the effect of a balance between protein synthesis and degradation in the protected and non-protected heart. Our data regarding the level of P-S6 provide evidences that after I/R injury the cell damage affects also the translation machinery, with a reduction of protein

production, and that the PostC procedure can rescue this damage. These data support the idea that ribosomal S6 subunit is a convergence point of protective signaling [41]. Moreover, Nrdp1 mRNA was upregulated after I/R injury, in line with literature data about Nrdp1 [13]. It has been shown that mouse model overexpressing Nrdp1 exhibit a downregulation of ErbB3 protein but only after I/R injury [13]. Nrdp1 degrades ErbB3 and our data suggest that this mechanism is perturbed after PostC treatment. In fact, we detected lower amount of Nrdp1 protein in PostC samples respect to I/R samples. Directly or indirectly mechanical PostC can influence ErbB3 protein levels, so we can assume that ErbB3 signalling pathway can be part of the protective signalling recruited by PostC treatment. It is well known that PostC activates PI3K-Akt pathway [42]. Actually, ErbB3 receptor has six docking sites for the binding of PI3K-p85 and this can result in a higher activation of PI3K-Akt pathway respect to the other ErbB receptors [43,44]. Zhang et al., observed in mice after coronary artery ligation that the phosphorylation of ErbB3 increases respect to the sham, meanwhile ErbB3 protein is unchanged [13]. At the time point analyzed we did not observe ErbB3 or AKT phosphorylation (data not shown), however we cannot rule out that it occurs in an earlier or later time.

In literature, data about Nrg1 expression in damaged heart are conflicting: in diabetic animals Nrg1 protein is reduced [40], while in human failing myocardium Nrg1 expression increases [39]. In animal models of heart failure, after an initial upregulation, Nrg1 level decreases, coincidently with the development of ventricular hypertrophy and pump failure [45].

In I/R scenario, cardioprotection by Nrg1 induced pharmacological conditioning has been described: *in vivo* Nrg1 pre-conditioning activates the protective PI3K/Akt pathway [46]. Similarly, post-conditioning with Nrg1, in both *in situ* and in isolated murine hearts, exerted a cardioprotective effect *via* PI3K/Akt pathway [47]. The same authors evidenced that Nrg1 activates this pathway only after I/R injury but not in sham samples. Therefore, we can argue that these protective effects involved also ErbB3 post-ischemic upregulation.

Recently D'Uva et al., demonstrated that transient expression of ErbB2 in adult mice heart after I/R injury results in a good functional and anatomical regeneration, and the authors postulated that probably treatment with Nrg1 avoids the loss of ErbB2 in cardiomyocytes or activates ErbB2 pathway [48]. The pivotal role of ErbB receptors in cell survival after hypoxia/reoxygenation has also been observed using zinc pyrithione, which

restores the basal zinc levels during I/R and prevents apoptosis by activating PI3K/Akt [49]. Since in our data ErbB3 mRNA is upregulated after I/R, we can argue that the cellular response to Nrg1 and its beneficial effects are mediated by ErbB2-ErbB3 heterodimer [50]. In fact, among ErbB receptors, only ErbB3 and ErbB4 can bind directly Nrg1. Our analysis revealed that Nrg1 mRNA does not change significantly after I/R or PostC, at the time point investigated. Nevertheless, in PostC ErbB3 protein is upregulated and a protective autocrine loop, involving physiological levels of Nrg1 and upregulated ErbB3, can occur.

To investigate the role of ErbB3 receptor in the context of redox stress, H9c2 cell model was used. The *in vitro* assays demonstrated that the expression of ErbB3 receptor in H9c2 cells can increase cell survival rate and can ameliorate mitochondrial resistance to oxidative stress. Nrg1 should stimulate an increase of cell survival rate in H9c2-ErbB3 cells, however H9c2-ErbB3 cells are protected also without exogenous Nrg1 addition, suggesting that ErbB3 is activated by the overexpression or by the endogenous Nrg1. Similarly to what seen for ErbB4 [32], our data suggest that oxidative stress activated ErbB3 signaling and that this receptor is involved in the protective adaptation to cardiac oxidative stress.

It is becoming more and more clear that Nrg1/ErbB system is a potential target for therapy in heart failure as highlighted by the promising results obtained in the clinical studies with Nrg1 administration [14,15]. However, the intrinsic mechanism of Nrg1 action is not completely understood; a better knowledge of Nrg1 and ErbB regulation in ischemia/reperfusion injury is now required to ameliorate the cure and increase the heart performance after acute myocardial infarction.

5. Conclusions

This is the first study emphasizing ErbB3 mRNA upregulation in the heart subjected to acute I/R challenge. Here, we show that PostC may counterbalance the effects of I/R on ErbB3 protein downregulation and that ErbB3 receptor transfection in cardiomyocytes inhibits death mechanisms activated by redox stress. These novel evidences of a pivotal role for ErbB3 in the context of I/R and redox biology highlights the importance of studies on these receptors not only for the optimization of Nrg1 treatment for heart diseases, but also for the development of new cancer therapies with no side effects on heart.

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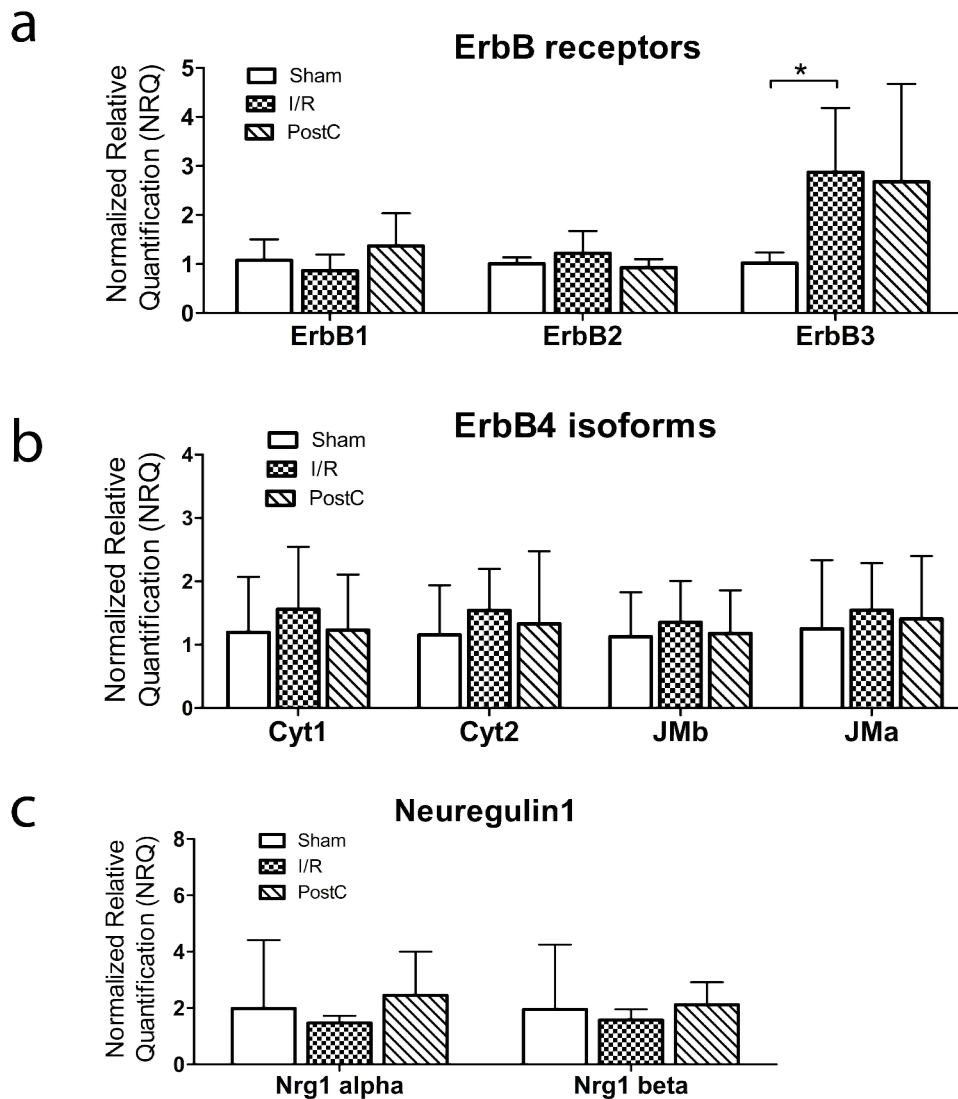


Fig. 1 ErbB3 mRNA is upregulated in Langendorff's model of heart after ischemia/reperfusion. The graphs show the results of quantitative real-time PCR analysis on heart apices after ischemia/reperfusion injury performed in Langendorff's model. **(a)** ErbB1 and ErbB2 transcription is not affected by the injury or by the postconditioning treatment at the time-point evaluated. ErbB3 transcription is upregulated in I/R. **(b)** All the ErbB4 isoforms are not perturbed by the ischemia/reperfusion injury. **(c)** No changes in transcription were detected for Neuregulin1, alpha or beta isoforms. Statistical analysis: One-way ANOVA was not significant. Student's t-Test analysis (comparison between Sham and I/R or Sham and PostC). *: $p < 0.05$.

Sham= control group; I/R= ischemia/reperfusion group; PostC= postconditioning group

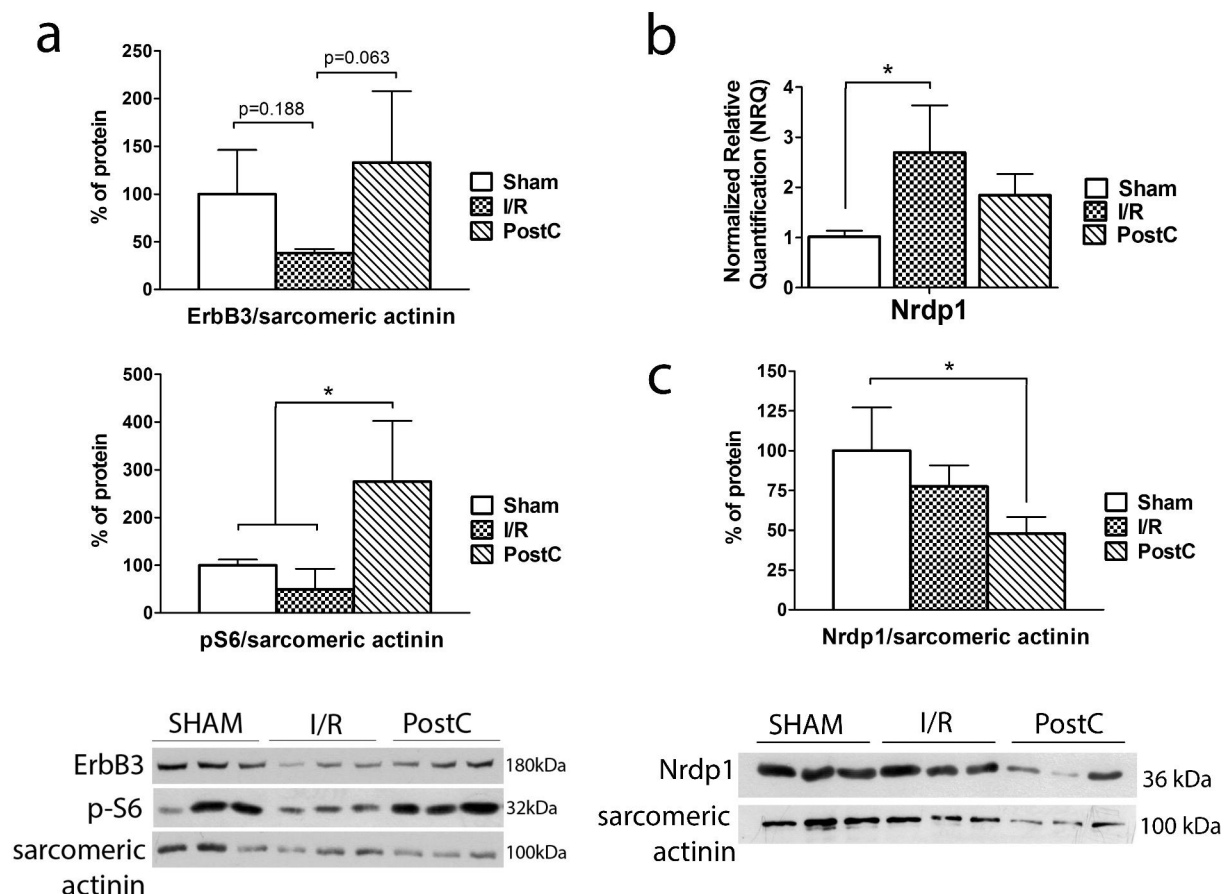


Fig. 2 Analysis of ErbB3, p-S6 protein and Nrdp1 mRNA and protein after ischemia/reperfusion injury with or without postconditioning treatment. (a) The graph shows the results of the protein quantification (technical triplicate) performed on heart apexes after ischemia/reperfusion injury; ErbB3 and p-S6 proteins were normalized to alpha sarcomeric actinin protein, using ImageJ program. About ErbB3 a tendency to downregulation can be detected for the I/R samples but not for PostC samples. p-S6 protein results upregulated in PostC samples compared to Sham (p=0.033) or I/R samples (p=0.012). The image shows a representative western blots. **(b)** The graph shows the results of quantitative real-time PCR analysis. Nrdp1 mRNA is upregulated in I/R group (p=0.011) respect to the control. **(c)** The results of a quantitative analysis (technical triplicate) of Nrdp1 protein are visible in the graph. Nrdp1 is downregulated in PostC group (p=0.041), but not in I/R group. The image shows representative western blot bands of Nrdp1 protein. Three animal's apexes were analysed for each group. Statistical analysis: One Way Anova plus Fisher's LSD test. * p<0.05. Sham= control group; I/R= ischemia/reperfusion group; PostC= postconditioning group

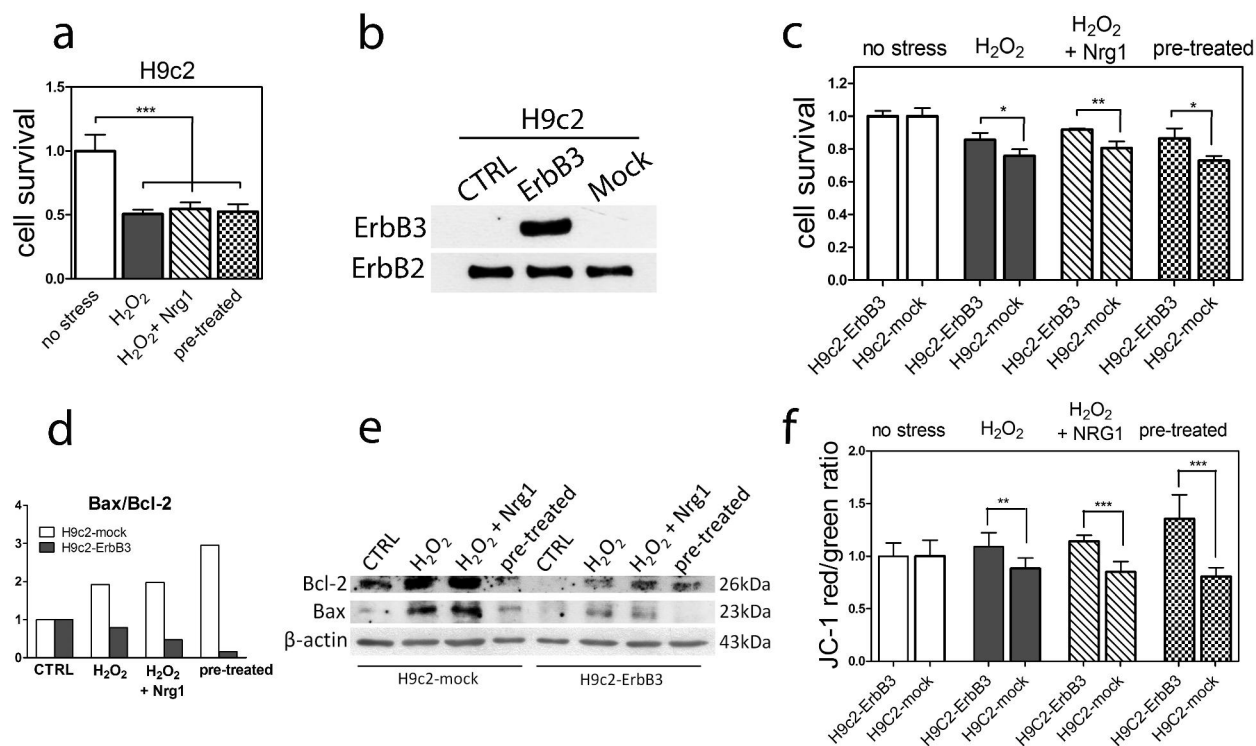


Fig. 3 Oxidative stress in H9c2 cells over-expressing ErbB3. **(a)** The graph shows the results of the thiazolyl blue tetrazolium bromide (MTT) assay in H9c2 cells exposed to oxidative stress (100 μ M H_2O_2 for 2 h). The addition of Nrg1 (50 ng/ml) in the medium immediately before (for 2 h) or concurrently to the oxidative stress does not affect cell survival. The assay was performed as technical seven-fold and biological triplicate. Statistical analysis refers to One Way Anova plus Bonferroni Post Hoc test. **(b)** Representative western blot bands, confirming the expression of ErbB3 protein in transfected H9c2 cells. **(c)** MTT analysis was performed to assess cell survival rate of H9c2 expressing ErbB3 exposed to oxidative stress. Four conditions were assayed (no stress, H_2O_2 , H_2O_2 with Nrg1, pre-treatment with Nrg1). H9c2-ErbB3 tolerate oxidative stress better than control cells (H9c2-mock). **(d)** The graph shows the ratio between Bax and Bcl-2 protein in H9c2-ErbB3 and -mock cells exposed to oxidative stress. **(e)** Representative western blot of 2 independent experiments is shown. **(f)** The graph illustrates the effects of oxidative stress on mitochondrial membrane potential in H9c2-mock and H9c2-ErbB3 cells, analyzed by JC-1 assay. All treatment values were normalized to mean value of no-stress group either in H9c2-ErbB3 and H9c2-mock cells. Statistical analysis: Student's t-Test analysis. *: $p < 0.05$; **: $p < 0.01$

Table 1. Primer sequences

GENE	Sequence Accession Number	FORWARD PRIMER	REVERSE PRIMER
ErbB1	U52529	5'-CACCACGTACCAGATGGATG-3'	5'-CGTAGTTTCTGGGGCATTTC-3'
ErbB2	NM_017003	5'-TGACAAGCGCTGTCTGCCG-3'	5'-CTTGATAGTGGGCGCAGGCTG-3'
ErbB3	NM_017218.2	5'-CGAGATGGGCAACTCTCAGGC-3'	5'-AGGTTACCCATGACCACCTCACAC-3'
ErbB4 JMa	AY375306.1	5'-GGACGGGCCATTCCACTTTACC-3'	5'-CCATGATGACCAGGATGAAGAGCC-3'
ErbB4 JMb	AY375308.1	5'-CATTGAAGACTGCATCGGCCTG-3'	5'-CCATGATGACCAGGATGAAGAGCC-3'
ErbB4 Cyt1	AY375306.1	5'-GGAATATTTGTCCCCCAGGCTTTC-3'	5'-GAGGAGGGCTGTGTCCAATTTAC-3'
ErbB4 Cyt2	AY375307.1	5'-GGAATATTTGTCCCCCAGGCTTTC-3'	5'-GTACACAACTGATTCCTATTGGAGTCAATTC-3'
Nrdp1	NM_001012195.1	5'-GATCCTTGAGTGGGTGAACTCCC-3'	5'-CTGCAGCACAGCGTCAGG-3'
Nrg1 alpha	AF194439	5'-GTGCGGAGAAGGAGAAAACCTTC-3'	5'-TTGCTCCAGTGAATCCAGGTTG-3'
Nrg1 beta	AF194438	5'-GTGCGGAGAAGGAGAAAACCTTC-3'	5'-AACGATCACCAGTAACTCATTTGG-3'
Ubc	NM_017314.1	5'-TCGTACCTTTCTCACCACAGTATCTAG-3'	5'-CCCATCACACCAAGAAACAAG-3'
HPRT	NM_012583.2	5'-GCCCCAAATGGTTAAGGTTGCAAG-3'	5'-ATCCAACAAAGCTGGCCTGTATCC-3'

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